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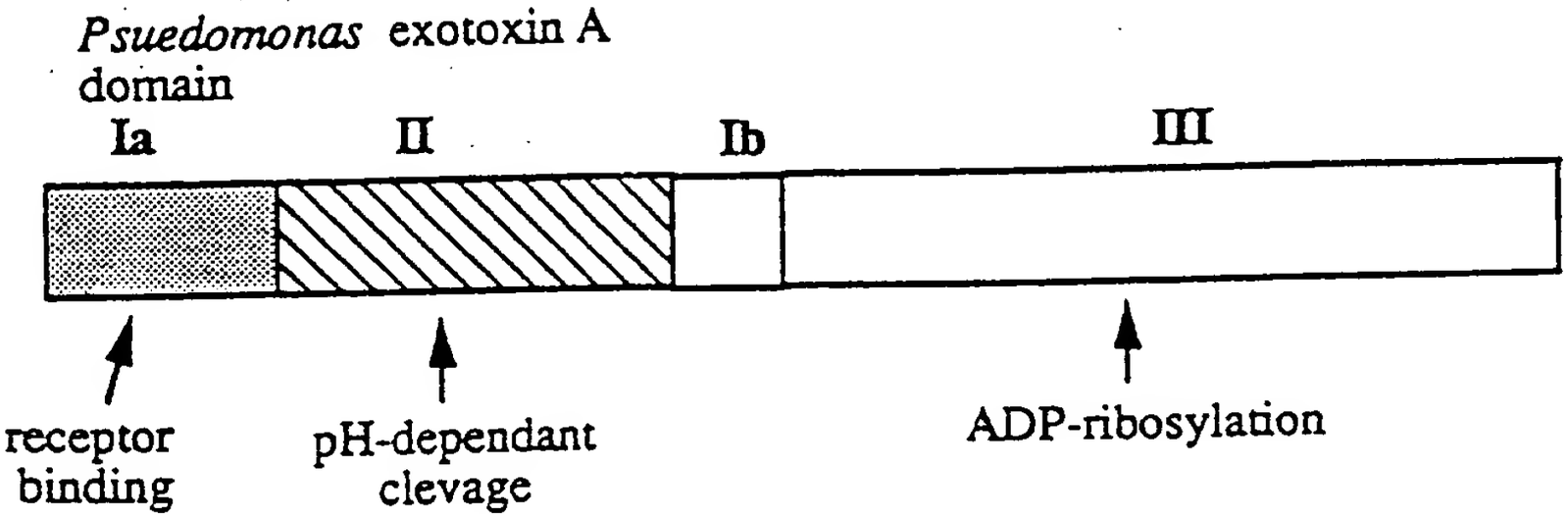

(57) Abstract

Methods for introducing proteins or nucleotide sequences into the nucleus using a novel receptor-mediated delivery system. The delivery system construct includes a cell receptor-binding domain, a cytoplasmic translocation domain, and a nuclear translocation signal domain. This system can transport functional macromolecule that will act once internalized into the nucleus.

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TITLE: TRANSLOCATION SIGNAL FACILITATED NUCLEAR DELIVERY OF MACROMOLECULES

FIELD OF THE INVENTION

The invention relates to methods of introducing foreign materials into a cell nucleus. More particularly, the present specification discloses methods for transporting nucleotide sequences or proteins into the nucleus using a novel translocation signal facilitated delivery system.

BACKGROUND

Individuals suffering from genetic diseases manifested by a deficiency in the expression of certain necessary proteins require augmentation with therapeutic doses of such proteins to lead normal lives. Currently, such treatment is mainly that of lifetime maintenance with periodic, exogenous introduction of the required protein. Such periodic treatment is cumbersome, expensive, and sometimes hazardous (such as for hemophiliacs who have a relatively high exposure rate to HIV).

It is desirable to utilize biomolecular manipulation to augment such protein deficiencies by introducing DNA (which code for the deficient proteins), and other nucleotide sequences and polypeptides (which regulate the expression of the deficient proteins) into the cells of patients suffering from genetic deficiencies. For example, such treatment involves the introduction of genes and regulators for factor VIII for hemophiliacs and al- antitrypsin for patients suffering from hereditary emphysema or adult respiratory distress syndrome (ARDS). Such approaches can even be extended to re-transform aberrant tumor cells in cancer patients. The most attractive manner for achieving such therapeutic transformation is to deliver a gene coding for the deficient gene product into the nucleus of somatic cells. In vitro delivery of foreign DNA into mammalian cells for gene expression has been achieved by three distinct approaches. The first approach takes advantage of the natural ability of viruses to infect cells and express viral DNA in the form of specific RNA and protein species (Cournoyer et al., 1991, "Gene transfer of adenosine deaminase into primitive human hematopoetic progenitor cells", Human Gene Therapy, 2:203; Rosenberg et al., 1990, "Gene transfer into humans: immunotherapy of patients with advanced melanoma using infiltrating lymphocytes modified by

retroviral gene transduction", New England Journal of Medicine, 323:570). In particular, advantage has been taken of mammalian retroviruses as vector systems that permit infection of a variety of cell types and allow for expression of many different foreign genes. Retroviruses and their recombinant forms are thought to bind to cells via specific receptors on the cell surface, after which they are internalized by endocytosis. Once endocytosed, the virus is able to evade the endosome-lysosome pathway by a mechanism which is thought to disrupt the endosome, escape degradation and permit entry into the cell nucleus.

A second approach fuses artificial lipophilic vesicles containing exogenous DNA with a cellular target (Felgner et al., 1987, "Lipofection: a highly efficient, lipid-mediated DNA- transfection procedure", Proc. Natl. Acad. Sci. USA, 84:7413). Delivery of DNA to the nucleus via lipophilic vesicle fusion is thought to be possible because it is hypothesized that endosome- associated degradation might be bypassed.

A third, non-specific approach for introduction of foreign DNA into cells is achieved by mixing exogenous DNA with a polycationic support, such as DEAE-dextran (McCutchan et al., 1968, "Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethyl aminoethyl-dextran", J. Natl. Cancer Inst., 41:351); or by complexing with calcium phosphate (Graham et al., 1973, "A new technique for the assay of infectivity of human adenovirus 5 DNA", Virology, 52:456).

The exogenous DNA mixture with polycationic support or calcium phosphate is then incubated with live cells (i.e. a transfection step). Uptake or endocytosis of DNA can be monitored by subsequent selection of the expressed phenotype, typically by complementation or by antibody-reactive surface markers. The mechanism of DNA uptake by the cells is largely unknown, but it is generally accepted that the DEAE-dextran or calcium phosphate protects the DNA from the nuclease activity of lysosomal cell compartments, followed by escape to the nucleus where expression occurs. Increased efficiency of expression using the transfection method can be achieved when lysosomotropic agents, such as chloroquine (Luthman et al., 1983, "High efficiency polyoma DNA transfection of chloroquine treated cells", Nucl. Acids Res., 11:1295), are included in the transfection mixtures. Lysosomotropic agents apparently reduce lysosomal destruction of DNA by increasing the relatively low pH necessary for activation of degradation.

Thus, several in vitro methods have been proposed for introduction of foreign DNA into mammalian cells. However, these known methods have inherent drawbacks in use. In order to translate the in vitro approaches of gene expression to the problem of delivering genetic material to the nucleus to express therapeutic amounts of gene product, many problems and considerations have to be addressed.

These problems include, but are not limited to the practical administration of a gene to an individual suffering from a particular disease amenable to therapy; targeting of the gene of interest to a particular cell-type or organ; efficient uptake of the gene by the cells; targeting of the gene to the nucleus; and efficient and sustained expression of the gene product. For example, while valuable as in vitro tools, retroviruses have considerable problems when used in vivo, including a very broad cell type specificity, the requirement for dividing cells to permit replication of the genome, inefficient expression of an inserted gene once in the nucleus, and questions of human safety.

Efforts have been made in the use of specific cell-surface receptors to mediate uptake of protein that is electrostatically- coupled to a piece of DNA that is capable of expressing a gene product of interest. This was first made possible by the observation that a complex consisting of orosomucoid-coupled poly-L-lysine could bind by salt bridges a plasmid DNA that coded for the bacterial-derived chloramphenicol acetyltransferase (CAT) gene. Presentation of this complex to cells permitted uptake by asialoglycoprotein receptors and short term expression of the CAT gene (Wu et al., 1989, "Evidence for targeted gene delivery to HepG2 hepatoma cells in vitro", Biochemistry, 27:887). However, the number of cells actually expressing the gene product of interest was several orders of magnitude lower than the number endocytosing the complex, suggesting considerable intra-cytoplasmic degradation of the complex after uptake.

Another approach was provided by the observation that after infection of a host cell, adenovirus, like other viruses, evades intracellular destruction and targets its genome to the cell nucleus (Curiel et al., 1991, "Adenovirus enhancement of, transferrin-polylysine-mediated gene delivery", Proc. Natl. Acad. Sci. USA, 88:8850). Advantage was taken of the ability of the adenovirus particle to "uncoat" its capsid proteins and permit early escape from the endosome-lysosome pathway. This group added adenovirus particles to a

mix of transferrin-poly-L-lysine that was electrostatically linked to DNA in the range of a few kilobases to nearly fifty kilobases to mediate enhanced expression of, in this case, the product of the luciferase gene (Cotten et al., 1992, "High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles", Proc. Natl. Acad. Sci. USA, 89:6094). Without added adenovirus capsids, expression of the luciferase gene was quite low, while with the adenovirus capsid, expression increased several orders of magnitude (Wagner et al., 1992, "Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes", Proc. Natl. Acad. Sci. USA, 89:6099). However, even with viral capsid uncoating, transport of transferrin-DNA complexes to the nucleus for expression is essentially random and introduces an element of uncertainty to such procedures.

A major disadvantage of the adenovirus-facilitated gene delivery technique is that success relies on the tedious preparation of chemically-derived protein-DNA-virus complexes of unknown quality to achieve the enhanced levels of expression reported. A further drawback is the requirement of the concomitant expression of both transferrin and adenovirus receptors on the desired cell targets.

SUMMARY OF THE INVENTION

The present invention is a translocation signal facilitated system for providing efficient, reproducible and targeted delivery of protein or DNA-protein complexes with therapeutic value, to the nuclei of mammalian cells. our approach takes advantage of the natural ability of some proteins, not just viruses, to enter cells and perform specific functions, such as directing their way out of endosomes into cytoplasm with the help of a cytoplasmic trans- location signal, and further directing their way to the nucleus with the help of a specific nuclear targeting signal.

The present disclosure demonstrates cell specific targeting and intracellular translocation with exotoxin A of *Pseudomonas aeruginosa* which generally infects patients at the site of surface injury, but which can also target its virulence at fibroblast cells and systemically, primarily to the liver, and secondarily to organs such as the kidney and spleen. Exotoxin A is composed of four domains, which are organized starting from the amino terminus as domains Ia, II, Ib, and III (Allured

et al.. 1986. "Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution". Proc. Natl. Acad. Sci. USA. 83:1320) (Figure 1).

Domain Ia (amino acids 1-252) binds the exotoxin specifically to a cell-surface receptor; domain II (amino acids 253-364) is the region of the exotoxin that is specifically cleaved after activation in the low pH compartment of the endosome and translocates the distal regions of the exotoxin out of the endosome; domain III (amino acids 405-613) contains the ADP- ribosylating activity of the toxin that inactivates ribosome associated protein elongation factor 2; domain Ib appears to be a structural, rather than functional, domain (amino acids 365-404). The features of the exotoxin protein which are important for each step in its uptake and delivery to the cell cytoplasm are described in Siegall et al., 1989, "Functional analysis of domains II, Ib and III of *Pseudomonas* exotoxin", J. Biol. Chem., 264:14256.

In addition, chimeric molecules can be constructed which allow attachment of other non-toxin-related receptor-binding domains to the ADP-ribosylation domain III for targeting toxin activity to the cytoplasm of certain cells for the purpose of cancer chemotherapy. It has also been demonstrated that exotoxin A domains Ia and II can be used to deliver an unrelated domain, in this case, a bacterial nuclease, barnase, to the cytoplasm of mammalian cells (Prior et al., 1992, "Translocation mediated by domain II of *Pseudomonas* exotoxin A: transport of barnase into the cytosol", Biochemistry, 31:3555).

We describe a novel and efficient system that overcomes the inadequacies of other systems for delivering both genes and proteins of therapeutic use to the nuclei of cells. A schematic representation of the molecular system designed for the nuclear targeting and delivery of DNA or proteins is depicted in Figure 2. This figure shows the preferered configuration of the critical domains needed f or targeting a macromolecule to the nucleus for therapeutic intervention.

The critical features of the present delivery system are "X" the receptor-binding domain; "II", the cytoplasmic translocation domain; "NTS", the nuclear translocation signal domain; and " Z", the functional macromolecule that will act once internalized into the nucleus. By way of example, Figure 3 shows the construction of a specific carrier protein and Figure 4 shows a schematic representation of the protein carrier-mediated delivery of a target protein to a cellular receptor for the exotoxin A binding domain. In the embodiment depicted in Figure 3, the receptor-binding domain ("X") is domain Ia derived from the

Pseudomonas exotoxin A gene, and the functional domain (" Z") is ß-galactosidase, the nuclear delivery of which is measured by the development of blue color in the presence of 5-bromo-4-chloro-3-indoyl-ß-D- galactopyranoside (X-gal) substrate. Starting from the amino terminus of the construct, the delivery system consists of domains Ia and II from *Pseudomonas* exotoxin A, a nuclear targeting signal (NTS) from SV40 T-antigen, and a functional version of 0-galactosidase. Domain Ia provides a natural means for directing the chimera to a particular cell type containing exotoxin A receptors such as fibroblasts, or to an organ such as the liver, by binding to a specific cell-surface receptor.

The protein-carrier complex is internalized into the cell by endocytosis, and the complex becomes engulfed in endosomes. During the course of its maturation, the pH of the endosome becomes more acidic, whereupon a pH-driven cleavage of domain II occurs, and it is the activation of this domain that is the key to the translocation of the distal portion of the protein (part of domain II, NTS, and ß-galactosidase) to the cytoplasm (see Figure 3). Once in the cytoplasm, the presence of a nuclear targeting signal (NTS), in this case derived from a viral protein of SV40 that is normally translated in the cytoplasm but functions in the nucleus, interacts with a cytoplasmic protein that promotes rapid translocation of the protein-carrier complex to the nucleus. Once in the nucleus, the polypeptide domain, in this case derived from ß-galactosidase, is free to function appropriately.

It will be appreciated by those skilled in the art that domain Ia could be substituted by other receptor-binding domains (for example, transforming growth factor alpha (TGF-α) ; or other toxin- derived ligands such as from diphtheria toxin) and that ß-galactosidase can be equally substituted by other protein domains, for example, transcription factors. In the latter case, it will be appreciated that when other protein domains that may naturally be found in the nucleus are substituted for ß-galactosidase, they may already have a nuclear translocation signal (NTS) coded within them, so that inclusion of additional NTS may be unnecessary. Similarly, one skilled in the art can appreciate that the SV40 nuclear translocation signal domain can be substituted with others such as yeast alpha-2, GAL 4, etc.

It will also be appreciated that neither the first nor the last protein domains need be contiguous polypeptide chains, but rather could be synthesized independently and attached to the amino- or

carboxyl- termini by chemical modification. It will be further appreciated that for the purpose of delivering a protein domain to the nucleus, non-protein receptor-binding domains could be substituted for domain Ia in the delivery system described above. Finally, in one embodiment the delivery of a chimeric molecule, such as above-described, can be targeted in vivo simply by injection into the systemic blood circulation.

For the purpose of delivering a nucleic acid, such as DNA, to the nucleus, the only modification of the chimeric construct described in Figure 2 is that domain " Z" is no longer a functional polypeptide domain, but rather a DNA-binding domain. This DNA- binding domain is attached by electrostatic binding either specifically or non-specifically to a piece of DNA, comprising either a sense or antisense oligonucleotide, or an expressible gene that includes replication, regulatory, transcriptional and/or translational sequence signals.

Figure 5 shows a pictorial representation of a protein carrier designed to deliver nucleic acid (DNA) to a cell nucleus for gene therapy. Figure 6 shows the construction of a protein carrier to be used for DNA binding, in which the receptor-binding domain ("X") is domain Ia derived from the *Pseudomonas* exotoxin A gene, domain II and the NTS domains are as described above, and domain "Z" is a stretch of poly-L-lysine which is used for electrostatic interaction with a plasmid DNA molecule that codes for galactosidase.

As described previously, domain Ia is used for binding of the delivery system-DNA complex molecule to a cellular receptor. After endocytosis and internalization, and maturation of the endosome, pH dependent cleavage of domain II occurs and translocation of the truncated complex to the cytoplasm ensues, where the NTS domains and their respective binding proteins transport the complex into the nucleus. The NTS domain binds to a cytoplasmic protein which mediates translocation of the complex to the nucleus. Once translocated, nuclear processes can unwind the DNA-binding domain from the DNA itself, followed by transcription and translation of RNAs coding for therapeutic proteins; if appropriate, replication of the targeted DNA may ensue.

It is understood that there are many other different examples of polypeptide domains that bind DNA including poly-L-lysine and poly-D-lysine, repeats of nuclear translocation signal sequences ("poly-NTS"), ornithine, putrescine, spermidine, spermine, histones and other non-

sequence-specific basic DNA-binding proteins, and sequence-specific DNA-binding proteins like homeobox domains. Those skilled in the art will appreciate that other polycationic macromolecules can be substituted as means for connecting DNA to the delivery system such as synthetic chemical linkers. With the exception of poly-L- (or -D-) lysine, other types of DNA-binding proteins often have NTS signals coded within them. In such cases, there would be no need for additional NTS domains in the final constructs. These DNA-binding domains can be contiguously translated as part of the chimeric molecule or attached to the basic construct by chemical modification. An alternative to the use of poly-L-lysine is to include within a DNA construct targeted for nuclear expression a short specific DNA sequence that could be bound by a specific DNA-binding protein, for example, a homeobox domain or other transcription factor. Free poly-L-lysine or poly-D-lysine can then be added to aid in the collapse of the DNA molecule itself to facilitate subsequent gene expression. As above, it will be recognized that the receptor-binding domain Ia of *Pseudomonas* exotoxin A can be substituted with other receptor-binding domains.

As the following Detailed Description outlines, the key to successful delivery of DNA or proteins to the nucleus pursuant to the present invention include (1) the use of *Pseudomonas* exotoxin A domain II or its functional equivalent to mediate translocation to the cytoplasm, and (2) the use of a nuclear targeting signal to mediate translocation to the nucleus.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLE 1

Cloning of *Pseudomonas* exotoxin A (ETA) gene

Genomic DNA is prepared according to the method described by Marmur et al., 1961, "A procedure for the isolation of deoxy-ribonucleic acid from micro-organisms", Journal of Molecular Biology, 3:208, from bacterial strain *Pseudomonas aeruginosa* PA103 (American Type Culture Collection 29260), originally described as producing exotoxin A (Liu, P., 1966, "The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced in vitro and in vivo", Journal of Infectious Diseases, 116:481).

Genomic DNA is cleaved with restriction endonucleases NotI and EcoRI, then is electrophoretically separated on a 0.8% low- melting

agarose gel, from which a region of ~2 to 2.6 kilobase pairs is excised (the expected size of the exotoxin A gene segment is 2.3 kb, Gray et al., 1984, "Cloning, nucleotide sequence, and expression in Escherichia coli of the exotoxin A structural gene of *Pseudomonas aeruginosa*", Proc. Natl. Acad. Sci. USA., 81:2645) and the DNA eluted. Purified NotI-EcoRI DNA is ligated to the phosphatased arms of bacteriophage ; λgt11 previously cleaved with NotI and EcoRI (Promega Corp., Madison, WI) in a reaction using T4 DNA ligase f or 16 hours at 15°C.

DNA is packaged into infectious particles using complementing packaging extracts obtained from Stratagene Corp. (San Diego, CA). Approximately 1000 recombinant λ particles are plated on indicator *Escherichia coli* Y1090 and lifted onto nitrocellulose filters. DNA is lysed in situ as described in Maniatis et al., 1982, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor, NY. Filters are then screened by hybridization with a DNA probe corresponding to the Ia domain of *Pseudomonas* exotoxin A (ETA), prepared by PCR amplification of cleaved *Pseudomonas aeruginosa* PA103 DNA using oligonucleotides GT105F (5'-GGATCCTCATGAGCGCCGAGGAAGCCTTCGACCTC) (SEQ. ID. NO. 1) and GT103R (5'-AAGCTTGGGAAAGTGCAGGCGATGACTGAT) (SEQ. ID. NO. 2) in the presence of digoxigenin-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Positive plaques are amplified for small-scale preparation of DNA which is recloned for easy manipulation into the Bluescript KS+ plasmid vector (Stratagene Corp., San Diego, CA). The 2.3 kilobase ETA genomic DNA is used as a source of template f or further amplifications and modifications of different ETA domains.

EXAMPLE 2

Cloning of *Pseudomonas* ETA domains Ia and II

The 2.3 kb DNA from Example 1 is used for polymerase chain reaction-mediated amplification of the binding (Ia) and cytoplasmic translocation (II) domains of the ETA gene, oligonucleotides GT105F (5'-GGATCCTCATGAGCGCCGAGGAAGCCTTCGACCTC) (SEQ. ID. NO. 1) and GT106R2 (5'-AAGCTTGGTGCCCTGCCGGACGAAGCGCT) (SEQ. ID. NO. 3) are used to amplify and for cloning DNA sequences coding for domains Ia and II, followed by downstream expression of the domains as non-secreted polypeptides in bacterial and insect cells.

Incorporated into the 5' ends of the oligonucleotides are restriction sites. BamHI and HindIII (underlined above) to facilitate downstream cloning and manipulation of domains. To achieve secretion of polypeptides having N-terminal domains Ia and II in the bacterial expression plasmid, pSE380 (Invitrogen Corporation, San Diego, CA), a second version of the clone is prepared using a forward oligonucleotide that primes within the DNA sequence coding for the Pseudomonas exotoxin A signal peptide sequence (GTO01SPF; TCATGATCCTGATACCCCATTGGATTCCCCTG) (SEQ. ID. NO. 4); for secretion in the insect cell expression system, a forward oligonucleotide coding within the signal sequence of an abundant baculovirus envelope glycoprotein, gp67 (Stewart et al., 1991, "Construction of an improved baculovirus insecticide containing an insect-specific toxin gene", Nature, 352:85) is used (GTO02SPF; GGATCCATGCTACTAGTAAATCAGTCACACCAAGGCTTCAATAAGGAACACACAAGC AAGATGGTAAGCGCTATTGTTTTATATGTGCTTTTGGCGGCGGCGGCGCATTCTGCCTTT GCGGAGGAAGCCTTCGACCTC) (SEQ. ID. NO. 5). Figure 7a shows the modified forms of domains Ia and II as a BamHI-BspHI/HindIII DNA cassette.

EXAMPLE 3

Preparation of Nuclear Translocation Signal

In order to facilitate targeting of proteins or protein-DNA complexes to the nucleus, a well-characterized nuclear translocation signal (NTS) domain from the mammalian virus, SV40 (Garcia-Bustos et al., 1991, "Nuclear protein localization" Biochemica et Biophysica Acta, 1071:83) is included in the preparation of constructs.

Two overlapping synthetic oligonucleotides with complementary 5' HindIII restriction sites are used to prepare the SV40 NTS domain, namely, GT108F; 5'-AGCTTCCTAAGAAGAAACGTAAGGTCA (SEQ. ID. NO. 6), and GT108R; 5'-AGCTTGACCTTACGTTTCTTCTTAGGA (SEQ. ID. NO. 7). Figure 7b shows amplified NTS domain produced by this method as a HindIII-HindIII cassette. This cassette is then ligated by T4 DNA ligase at the 3' end of ETA domains Ia and II. The position of the HindIII-Hind III SV40 NTS cassette relative to ETA IA-II domain cassette is shown in Figure 7d.

EXAMPLE 4

Cloning of *Escherichia coli* lac z gene

As a means of visually detecting proteins that are targeted to the nucleus. constructs are prepared that include an amino-terminal truncated version of the bacterial lac Z ('lac Z) gene coding for ß-galactosidase. This DNA segment was obtained by amplification of the lac Z gene-containing plasmid, pCH110 (Pharmacia. Piscataway. NJ). using oligonucleotides GT107F (5'- AAGCTTCAACGTCGTGACTGGGAAAACCCTGGCGTT) (SEQ- ID. NO. 8) and GT107R (5- CTGCAGCTATTATTTTTGACACCAGACCAACTGGTAATG) (SEQ. ID. NO. 9). Figure 7c shows the 'lac Z gene sequence as a HindIII-PstI cassette.

EXAMPLE 5

Construction of a Protein Carrier for Nuclear Delivery of a Target Protein: ETA domains Ia and II/ NTS / ß-galactosidase

Figure 7d shows the assembly of DNA cassettes coding for *Pseudomonas* ETA domains Ia and II. the SV40 NTS domain. and the 'lac Z gene. The ETA IA-II DNA cassette is ligated to the NTS and lac Z DNA cassettes by T4 DNA ligase using the common HindIII restriction sites that are included within the PCR amplification primers. The final DNA construct is inserted into plasmid vectors for expression in the baculovirus system using one of several baculovirus expression plasmids (for example. PVL1393: Webb et al.. 1990. "Expression of proteins using recombinant baculoviruses". Technique - A Journal of Methods in Cell and Molecular Biology. 2:173). or by expression in a bacterial host by incorporation into a high expression plasmid vector. like pET. which uses T7 promoter-specific expression (Studier et al.. 1986. "Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned gene". J. Mol. Biol.. 189:113).

Protein constructs expressed in either baculovirus or bacteria are purified from cell lysates and/or media over anti-ß- galactosidase antibody columns linked via protein A. Proteins thus prepared are suitable for targeting for targeting functional ß- galactosidase to the nucleus by in vitro incubation with exotoxin A-sensitive mammalian cells (for example. L-M cells. American Type Culture Collection CCL 1.2 or Chang liver cells. American Type Culture Collection CCL 13) and/or after injection into the mammalian blood circulation for in vivo targeting to either the liver and/or secondary organ sites.

Successful nuclear targeting is assayed histochemically by the conversion of the colorless X-gal substrate to blue. indicating functional

ß-galactosidase. Similar constructs can be prepared for use with other domains substituted for ß-galactosidase and/or ETA Ia domains.

EXAMPLE 6

Construction of ETA domains Ia and II/ NTS / cationic polypeptide

Figure 8 shows the preferred configuration for a continuous polypeptide that has a polycation stretch at the "Z" domain. In this example, we use poly-L-lysine as the polycation stretch. A polylysine polypeptide segment is generated from a synthetic DNA segment of ~200 - 300 bases of poly AAA/AAG (lysine codons) containing an HindIII restriction site at its 5' end and a PstI site at its 3' end (as in 'lac Z). Just proximal to the PstI site is placed a stop codon like TAG, TGA or TAA to terminate translation.

As an alternative to the use of polylysine, a DNA segment containing as many as 20-30 NTS repeats derived from the SV40 sequence (since the SV40 nuclear targeting signal is polycationic), or a smaller number of repeats if they are derived from other longer nuclear targeting signals, can be used (Figure 9). Any one of these different polycationic segments can be ligated at the 3' end of the core construct consisting of ETA IA-II/ NTS (BamHI-HindIII-HindIII) cassette (refer to Figure 7d).

As a further alternative to incorporating polycation substitutions for the ß-galactosidase domain as continuously translated polypeptides, it will be appreciated that polylysine, for example, can be covalently coupled by chemical modification to a bacterial or baculovirus -expressed ETA IA-II-NTS core construct in order to achieve a similar effect (Figure 10).

EXAMPLE 7

Construction of ETA domains Ia and II/ NTS / DNA binding protein

Figure 11 shows an alternative method for preparing a DNA binding protein domain (domain "Z"). This polypeptide domain is derived from any of several nuclear proteins that bind specific DNA sequences, for example, homeobox domains or yeast GAL4 protein. These domains are amplified by the PCR from genomic DNA or from cDNA coding for these domains. Included at the 5' and 3' ends of the amplified domain are the HindIII and PstI restriction sites that are also used for

constructing 'lac Z or polylysine domains. In our example. a short defined DNA sequence is included in the construction of a DNA gene that will be targeted for expression in the nucleus. one or more of these domains can be used to bind DNA: subsequent complex formation would include addition of non-covalently added basic proteins or polycations, like poly-L-lysine (or poly-D-lysine).

EXAMPLE 8

Construction of a Protein Carrier-DNA Complex for Nuclear Delivery of DNA for expression: ETA domains Ia and II/ NTS / poly-L-lysine linked to a plasmid coding for ß-galactosidase

Figure 12 shows the preferred method for preparing a protein carrier that can deliver a DNA construct to the nucleus of a mammalian cell for the purpose of expressing a gene coding for ß- galactosidase. The ETA IA-II domains described above are ligated to the NTS domain by T4 DNA ligase using the common HindIII restriction sites that are included within the amplimers. This DNA construct is inserted into plasmid vectors for expression in the baculovirus system using one of several baculovirus expression plasmids (for example, pVL1393; Webb et al., 1990, "Expression of proteins using recombinant baculoviruses", Technique - A Journal of Methods in Cell and Molecular Biology, 2:173), or by expression in a bacterial host by incorporation into a plasmid vector, like pET, which uses T7 promoter-specific expression (Studier et al., 1986, "Use of bacteriophage T7 RNA polymerase to direct selective high- level expression of cloned gene", J. Mol. Biol., 189:113).

Expressed protein carriers are purified from cell lysates and/or media by conventional ion exchange chromatography over an anionic exchange column such as carboxymethyl-Sepharose, that will bind basic proteins. Commercially available poly-L-lysine (40, 000 mol. wt.) is then coupled to the ETA IA-II / NTS domain by using conventional NHS (N-hydroxysuccinimide) chemistry. Uncomplexed poly-L-lysine is removed by chromatography over Sephadex G-100 (or its equivalent) in phosphate-buffered saline.

The resultant purified protein carrier is then incubated with pCH110, a commercially available mammalian expression plasmid coding for ß-galactosidase (Pharmacia, Piscataway, NJ), in 2M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and diluted down to 150 mM NaCl by dialysis. The formation of protein-DNA complexes is determined by gel

electrophoresis on 1% agarose, where plasmid DNA alone migrates in its supercoiled form at a relative molecular marker weight position of 7.2 kb, while complexes of supercoiled DNA with the ETA Ia-II/NTS/poly-L-lysine migrate at the top of the agarose gel, indicating very high molecular weight complexes. Uncomplexed DNA is separated by size exclusion chromatography on Sepharose gel bead matrices. Protein carrier-DNA complexes prepared in this way are suitable for targeting to the nucleus by in vitro incubation with exotoxin A-sensitive mammalian cells (for example, L-M cells, American Type Culture Collection CCL 1.2 or Chang liver cells, American Type Culture Collection CCL 13) and/or after injection into the mammalian blood circulation for in vivo targeting to liver and/or secondary organ sites.

Successful nuclear targeting can then be assayed histochemically by the conversion of the colorless X-gal substrate to blue, indicating functional ß-galactosidase.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Miles Inc.

(Thomas R. Barnett and Rathindra C. Das)

(ii) TITLE OF INVENTION: TRANSLOCATION SIGNAL FACILITATED NUCLEAR DELIVERY OF MACROMOLECULES

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Miles Inc.
(B) STREET: 400 Morgan Lane
(C) CITY: West Haven
(D) STATE: Connecticut
(E) COUNTRY: USA
(F) ZIP: 06516

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy diskette
(B) COMPUTER: IBM PC
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: Word Perfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Barbara A. Shiemi, Esq.
(B) REGISTRATION NUMBER: 29,862
(C) REFERENCE/DOCKET NUMBER: MWH 314

(viii) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (203) 937-2340
(B) TELEFAX: (203) 937-2795

(2) INFORMATION FOR SEQUENCE ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:
Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Gray et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 81
(D) PAGE: 2645
(E) DATE: 1984

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 1

GGATCCTCAT GAGCGCCGAG GAAGCCTTCG ACCTC 35

(3) INFORMATION FOR SEQUENCE ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Gray et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 81
(D) PAGE: 2645
(E) DATE: 1984

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 2

AAGCTTGGGA AAGTGCAGGC GATGACTGAT 30

(4) INFORMATION FOR SEQUENCE ID NO: 3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Gray et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 81
(D) PAGE: 2645
(E) DATE: 1984

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 3

AAGCTTGGTG CCCTGCCGGA CGAAGCGCT 29

(5) INFORMATION FOR SEQUENCE ID NO: 4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Gray et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 81
(D) PAGE: 2645
(E) DATE: 1984

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 4

TCATGATCCT GATACCCCAT TGGATTCCCC TG 32

(6) INFORMATION FOR SEQUENCE ID NO: 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 138 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Stewart et al.
(B) JOURNAL: Nature
(C) VOLUME: 352
(D) PAGE: 85
(E) DATE: 1991

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 5

GGATCCATGC TACTAGTAAA TCAGTCACAC CAAGGCTTCA 40
ATAAGGAACA CACAAGCAAG ATGGTAAGCG CTATTGTTTT 80
ATATGTGCTT TTGGCGGCGG CGGCGCATTC TGCCTTTGCG 120
GAGGAAGCCT TCGACCTC 138

(7) INFORMATION FOR SEQUENCE ID NO: 6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Benditt et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 86
(D) PAGE: 9327
(E) DATE: 1989

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 6

AGCTTCCTAA GAAGAAACGT AAGGTCA 27

(8) INFORMATION FOR SEQUENCE ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Benditt et al.
(B) JOURNAL: Proc. Natl. Acad. Sci. USA
(C) VOLUME: 86
(D) PAGE: 9327
(E) DATE: 1989

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 7

AGCTTGACCT TACGTTTCTT CTTAGGA 27

(9) INFORMATION FOR SEQUENCE ID NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Kalnins et al.
(B) JOURNAL: EMBO J.
(C) VOLUME: 2
(D) PAGE: 593
(E) DATE: 1983

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 8

AAGCTTCAAC GTCGTGACTG GGAAAACCCT GGCGTT 36

(10) INFORMATION FOR SEQUENCE ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 nucleotides
(B) TYPE: nucleic acids
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE:

Other nucleic acid - oligonuleotide primer

(iii) PUBLICATION INFORMATION:

(A) AUTHORS: Kalnins et al.
(B) JOURNAL: EMBO J.
(C) VOLUME: 2
(D) PAGE: 593
(E) DATE: 1983

(iv) SEQUENCE DESCRIPTION: SEQ ID NO: 9

CTGCAGCTAT TATTTTTGAC ACCAGACCAA CTGGTAATG 39

We Claim:

1. A composition comprising a polypeptide which contains a receptor-binding domain, a cytoplasmic translocation domain, a nuclear translocation domain, and a means for connecting a selected macromolecule to the said polypeptide.

2. The composition of Claim 1 wherein the said macromolecule is selected from the group consisting of nucleotides, oligopeptides, polypeptides, and proteins, a nucleotide sequence that encodes for factor VIII, a nucleotide sequence that encodes for alpha-1-antitrypsin, a polypeptide which is a regulator of gene expression, Beta-galactosidase.

3. The composition of Claim 1 wherein the said receptor-binding domain is a toxin-derived ligand for a specific cell receptor, is derived from diphtheria toxin, or from Pseudomonas exotoxin A.

4. The composition of Claim 1 wherein the said cytoplasmic translocation domain is derived from Pseudomonas exotoxin A.

5. The composition of Claim 1 wherein said nuclear translocation signal domain is selected from the group consisting of SV40 nucleic acid sequence, yeast alpha-2 nucleic acid sequence, and GAL-4 nucleic acid sequence.

6. The composition of Claim 1 wherein the means for connecting the macromolecule to the nuclear translocation domain is a polycationic macromolecule which is selected from the group consisting of poly-L-Lysine, poly-D-Lysine, poly NTS, ornithine, putrescine, a histone, GAL 4, a homeobox domain, spermidine, and spermine.

7. A method for inserting an exogenous macromolecule into a target cell nucleus comprising the steps of:

a) administering a polypeptide which contains a receptor-binding domain, a cytoplasmic translocation domain, a nuclear translocation domain, and a means for connecting a selected macromolecule to the said polypeptide, to target cells,

b) incubating cells with said polypeptide, and

c) determining transfer by an assay.

8. The method as in Claim 7 wherein the polypeptide is as described by Claim 2, Claim 3, Claim 4, Claim 5, or Claim 6.

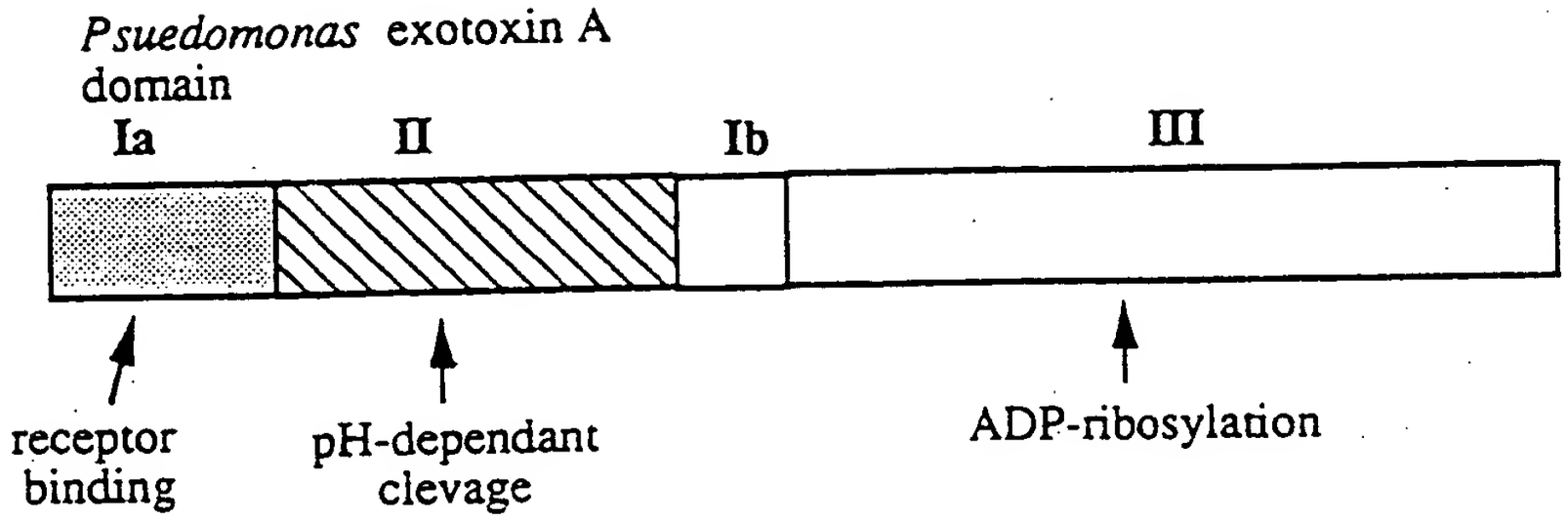


Figure 1

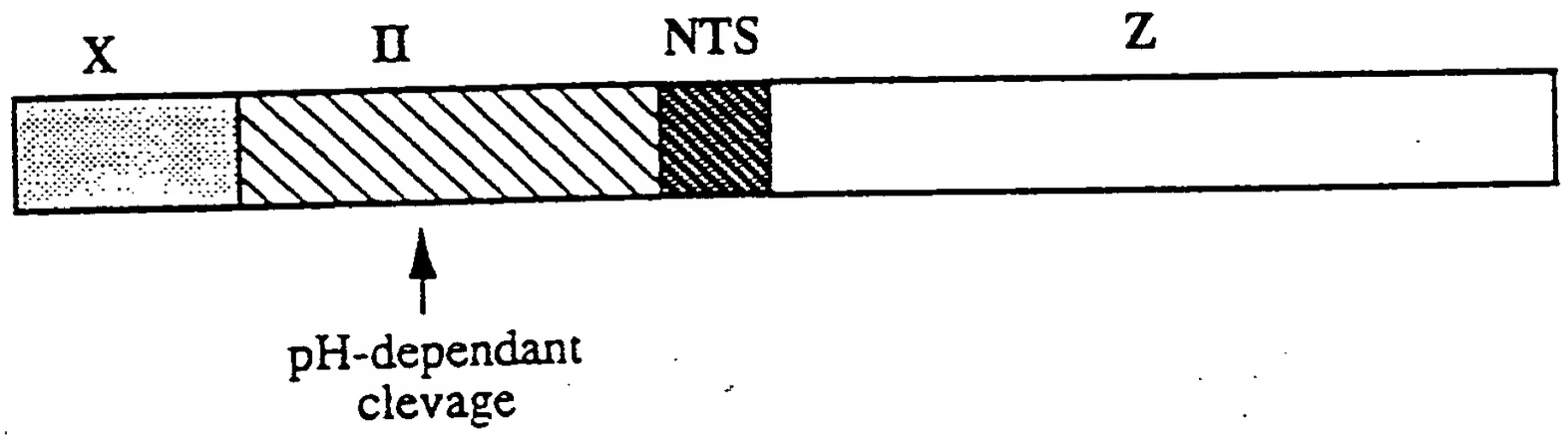


Figure 2

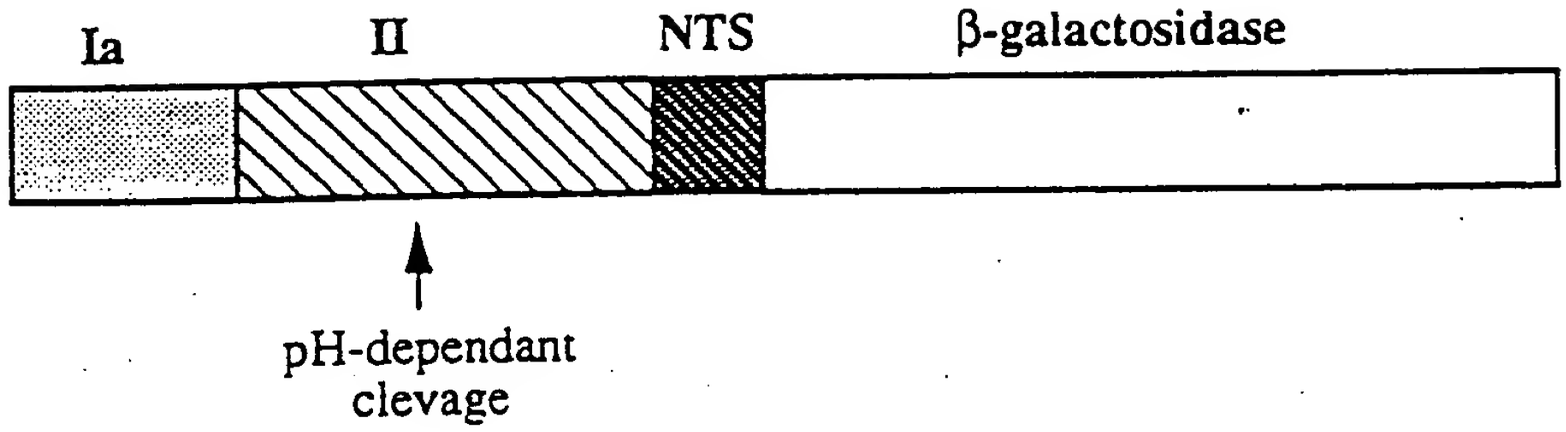


Figure 3

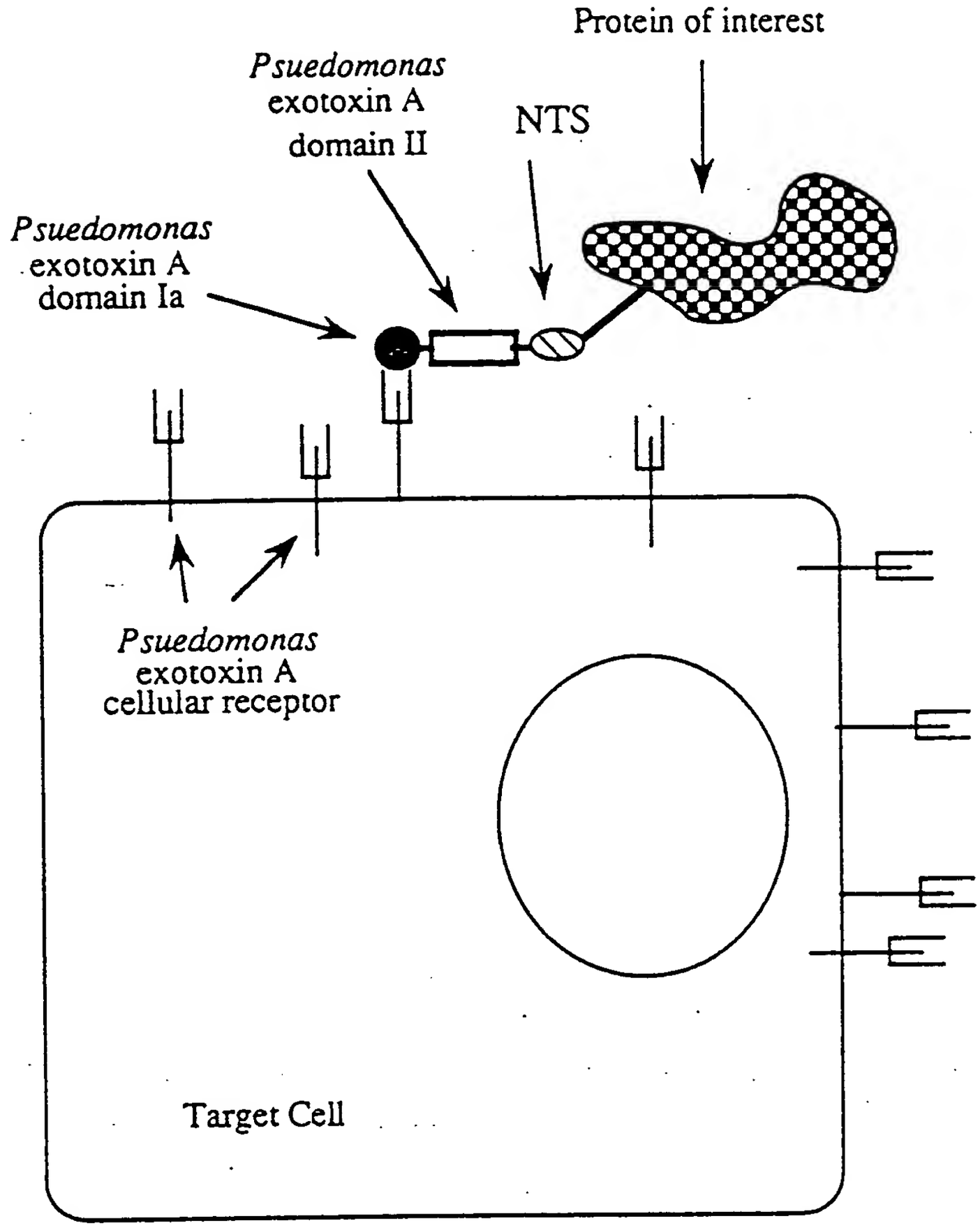


Figure 4

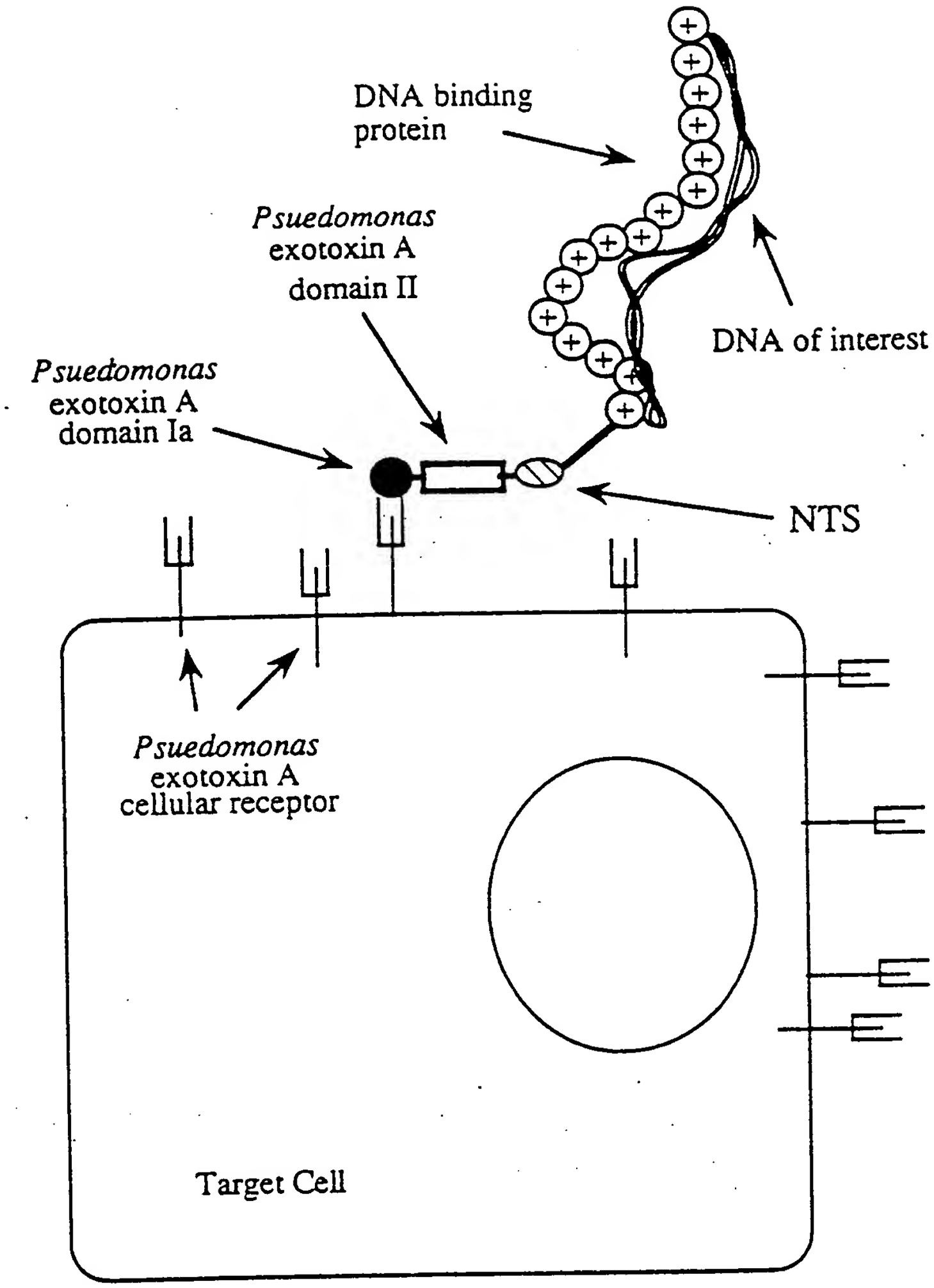

DNA binding protein
Psuedomonas exotoxin A domain II
Psuedomonas exotoxin A domain Ia
DNA of interest
NTS
Psuedomonas exotoxin A cellular receptor
Target Cell

Figure 5

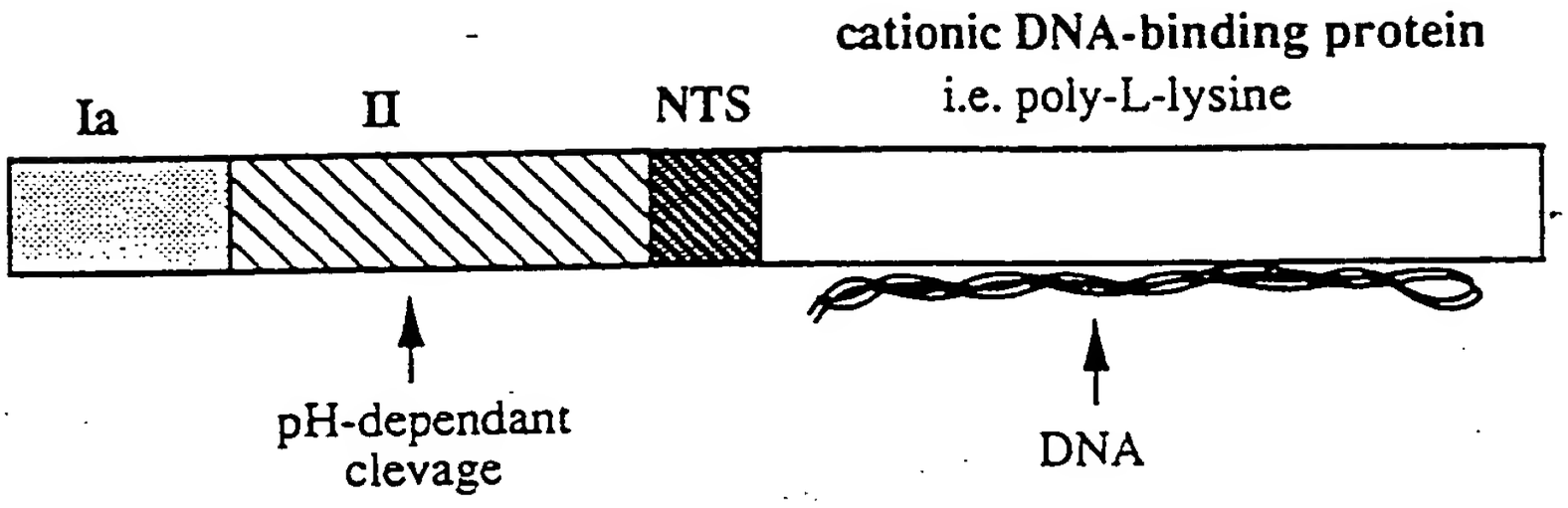


Figure 6

Figure 7 a

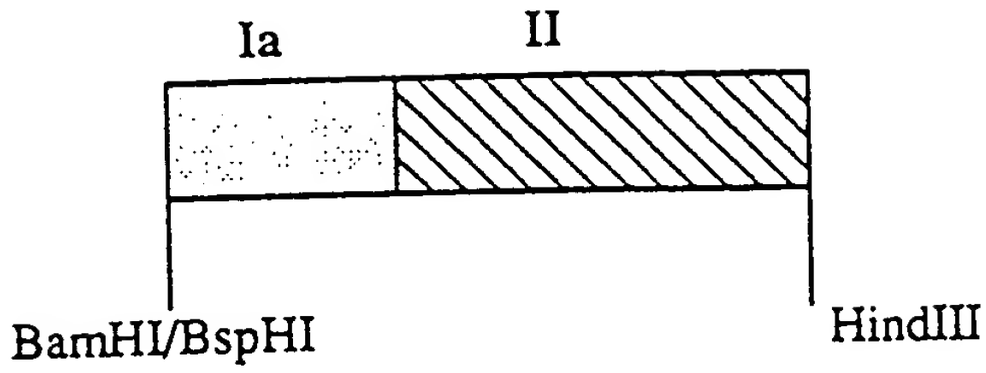


Figure 7 b

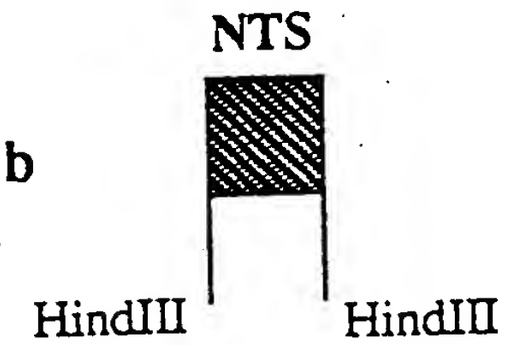


Figure 7 c

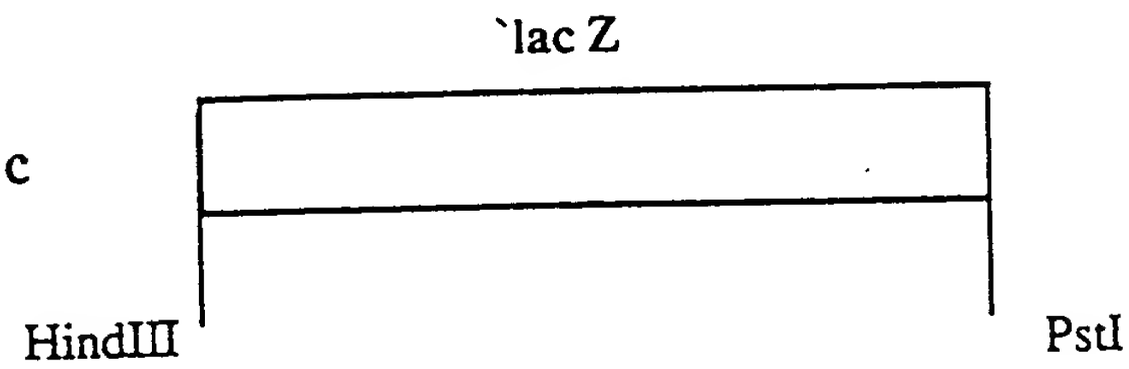
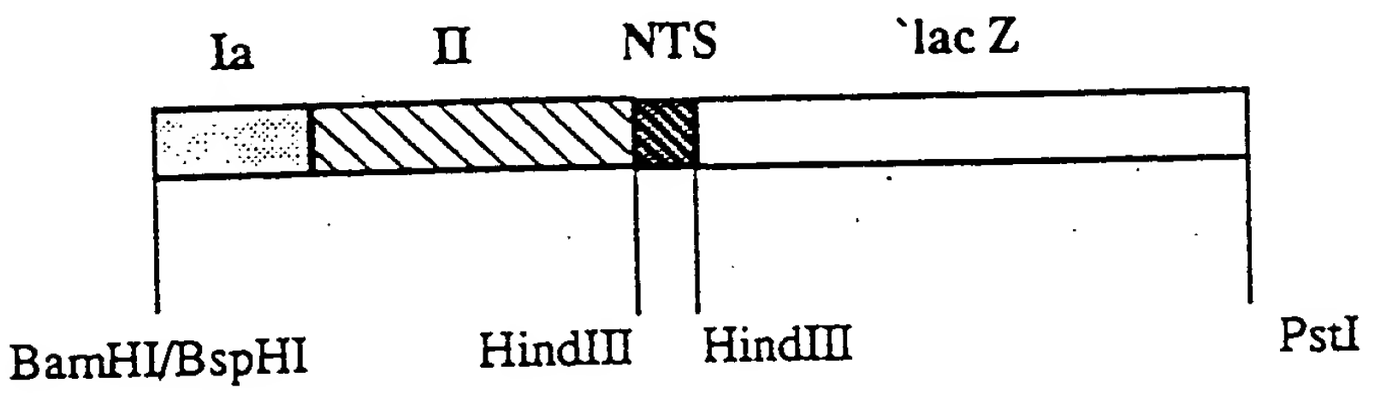


Figure 7 d



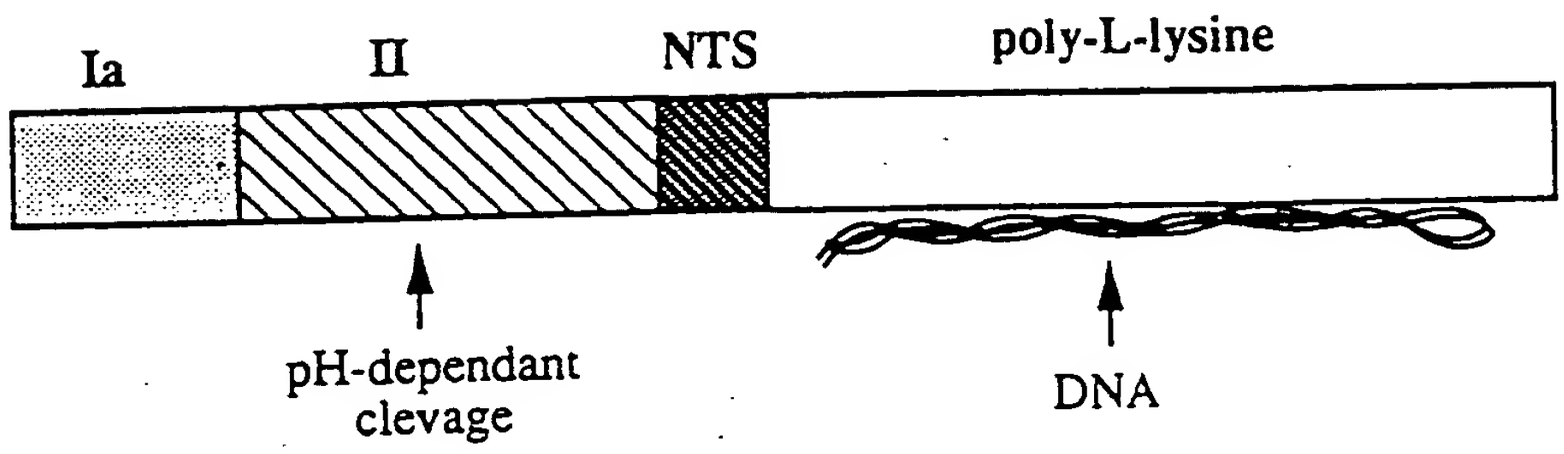


Figure 8

II

HindIII

poly-NTS

DNA

-L-lysine

PstI

e 9

poly-L-lysine

indIII

PstI

12

2

E SHEET

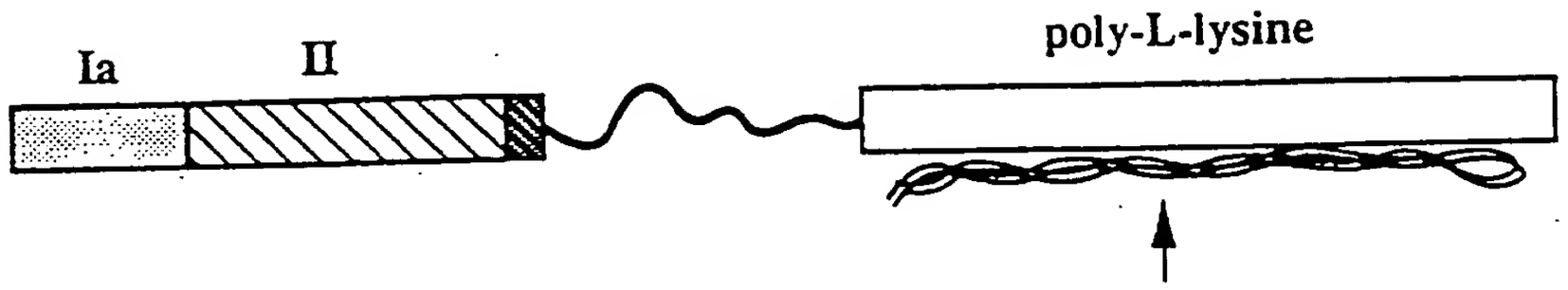


Figure 10

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/87 C12N15/31 C12N15/62 C07K13/00 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY vol. 31, no. 14 , 14 April 1992 , AM. CHEM. SOC.,WASHINGTON,DC,US; pages 3555 - 3559 T.I. PRIOR ET AL. 'Translocation mediated by domain II of Pseudomonas exotoxin A: Transport of barnase into the cytososl' cited in the application see page 3555, left column, line 11 - line 15; figures 1,3 see page 3558, right column, line 23 - page 3559, left column, line 1 see page 3559, left column, line 24 - line 30 --- -/--	1,3,4,6, 7

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1993	06 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. BIOL. CHEM. vol. 262, no. 10 , 5 April 1987 , AM. SOC. MOL. BIOL., INC.,BALTIMORE, US; pages 4429 - 4432 G.Y. WU AND C. H. WU 'Receptor-mediated in vitro gene transformation by a soluble DNA carrier system' see page 4431, right column, line 32 - page 4432, left column, line 12 ---	1,3,4,6, 7
Y	PROC. NATL. ACAD SCI. vol. 89, no. 13 , 1 July 1992 , NATL. ACAD SCI., WASHINGTON, DC, US; pages 6099 - 6103 E. WAGNER ET AL. 'Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes' cited in the application see page 6102, right column, line 1 - page 6103, right column, line 5 ---	1,3,4,6, 7
A	J. BIOL. CHEM. vol. 267, no. 2 , 15 January 1992 , AM. SOC. MOL. BIOL., INC.,BALTIMORE, US; pages 963 - 967 J.M. WILSON ET AL. 'Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits' the whole document ---	1-8
A	WO,A,92 05250 (UNIVERSITY OF CONNECTICUT) 2 April 1992 the whole document ---	1-8
A	WO,A,91 09958 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 11 July 1991 the whole document ---	1-8
P,Y	EP,A,0 544 292 (BOEHRINGER MANNHEIM GMBH) 2 June 1993 the whole document ---	1,3,4
T	WO,A,93 17102 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 2 September 1993 the whole document ---	1-8
T	WO,A,93 18759 (BAYLOR COLLEGE OF MEDICINE) 30 September 1993 the whole document -----	1-8

2
1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9205250	02-04-92	AU-A-	8628291	15-04-92
		CA-A-	2092319	26-03-92
		EP-A-	0556197	25-08-93
WO-A-9109958	11-07-91	AU-A-	7182991	24-07-91
		CA-A-	2071214	22-06-91
		EP-A-	0506884	07-10-92
EP-A-0544292	02-06-93	DE-A-	4139001	03-06-93
WO-A-9317102	02-09-93	NONE		
WO-A-9318759	30-09-93	NONE		